The p11 Subunit of the Annexin II Tetramer Plays a Key Role in the Stimulation of t-PA-Dependent Plasminogen Activation†

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ABSTRACT: Annexin II tetramer (AIIt) is an important endothelial cell surface protein receptor for plasminogen and t-PA. AIIt, a heterotetramer, is composed of two p36 subunits (called annexin II) and two p11 subunits. In this report, we have compared the ability of the isolated p36 and p11 subunits to stimulate t-PA-dependent [Glu]plasminogen activation. The fluid-phase recombinant p11 subunit stimulated the rate of t-PA-dependent activation of [Glu]plasminogen about 46-fold compared to an approximate stimulation of 2-fold by the recombinant p36 subunit and 77-fold by recombinant AIIt. The stimulation of t-PA-dependent activation of [Glu]plasminogen by the p11 subunit was Ca^{2+}-independent and inhibited by ϵ-aminocaproic acid. [Glu]Plasminogen bound to a p11 subunit affinity column and could be eluted with ϵ-aminocaproic acid. Both AIIt and the p11 subunit protected t-PA and plasmin from inactivation by PAI-1 and α_2-antiplasmin, respectively. A peptide to the C terminus of the p11 subunit (85-Y-F-V-V-H-M-K-Q-K-G-K-K-96) inhibited the p11-dependent stimulation of t-PA-dependent plasminogen activation. In addition, a deletion mutant of the p11 subunit, missing the last two C-terminal lysine residues, retained only about 15% of the activity of the wild-type p11 subunit. Similarly, a mutant AIIt composed of the wild-type p36 subunit and the p11 subunit deletion mutant possessed about 12% of the wild-type activity. These results, therefore, suggest that the C-terminal lysine residues of the p11 subunit bind plasminogen and participate in the stimulation of t-PA-dependent activation of plasminogen by AIIt.

Annexin II tetramer (AIIt)[1] is a Ca^{2+}- and phospholipid-binding protein that can exist intracellularly and on the extracellular surface of the plasma membrane (reviewed in refs *1* and *2*). AIIt is a heterotetrameric complex that is composed of two p36 subunits and two p11 subunits ($p36_2$-$p11_2$). The p36 subunit, which is also referred to as annexin II, can exist as a subunit of AIIt or as an isolated monomer. However, the complexed form of annexin II is the predominant form in most cells (reviewed in ref *1*). For example, in MDCK cells, bovine intestinal epithelial cells, and calf pulmonary artery endothelial cells, 90−95% of the total cellular annexin II is present as a subunit of AIIt (*3*, *4*).

Annexin II contains three distinct functional regions: the N-terminal region, the C-terminal region, and the core region (*5*−*7*). The core region of annexin II contains the Ca^{2+}- and phospholipid-binding sites. The C-terminal region contains the 14-3-3 homology domain, the plasminogen binding domain (*8*), and the heparin binding domain (*9*). The N-terminal region of annexin II contains two important regulatory domains, the P and the L domains. The P domain contains the phosphorylation sites for protein kinase C (Ser-25) and $pp60^{src}$ (Tyr-23). Phosphorylation of these sites inhibits many of the in vitro activities of the protein (*10*, *11*). The L domain, consisting of the first 14 amino acid residues of the N terminus, contains a high-affinity binding site for the p11 subunit (reviewed in ref *1*).

The biochemical properties of annexin II and the annexin II present as the p36 subunit of AIIt are distinct (*12*, *13*). Several laboratories have reported that the binding of p11 to annexin II produces a conformational change in annexin II which influences its interaction with several of its ligands (*5*, *12*, *14*, *15*). Similarly, we found that the binding of recombinant p11 to recombinant annexin II results in a decrease in the $A_{0.5}(Ca^{2+})$ for chromaffin granule aggregation from 0.23 mM for recombinant p36 to 1.0 μM for the recombinant AIIt. We also found that the binding of recombinant p11 to recombinant p36 results in a decrease in the $A_{0.5}(Ca^{2+})$ of phospholipid liposome aggregation from 0.83 μM for recombinant p36 to 0.26 μM for recombinant AIIt. In the case of the F-actin bundling activity of AIIt, the role of p11 binding is most dramatic. Individually, p11 and annexin II are incapable of significant bundling of F-actin; however, the p36 subunit of AIIt can participate in

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[1] Abbreviations: BSA, bovine serum albumin; AIIt, annexin II tetramer; EACA, ϵ-amino-*n*-caproic acid; p11, p11 light chain of the annexin II tetramer; p36, p36 subunit of the annexin II tetramer (annexin II); t-PA, tissue-type plasminogen activator; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PAI-1, plasminogen activator inhibitor type 1; *p*NA, *p*-nitroaniline; HUVEC, human umbilical vein endothelial cells; U, units for expression of the initial rates of plasmin generation (A_{405nm}/min^2 × 10^3); buffer A, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM $CaCl_2$; $p11_{del-KK}$ subunit, p11 subunit in which the last two C-terminal lysine residues have been deleted; PVDF, polyvinylidene difluoride.

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the Ca^{2+}-dependent formation of anisotropic F-actin bundles (*3*, *13*, *15*). Therefore, a wealth of evidence has suggested that p11 modulates the activities of the p36 subunit and, therefore, plays the role of a regulatory subunit in AIIt.

The mechanism by which AIIt is exported to the extracellular surface is unclear. AIIt subunits lack signal peptide sequences and therefore cannot go through the conventional endoplasmic reticulum secretory pathway (*2*). However, there are a number of other proteins that lack signal peptide sequences that are also secreted; these include fibroblast growth factor, interleukin-1, and galectins. Annexins can also be secreted. For example, annexin V has been shown to be secreted by the prostate gland (*16*, *17*). Annexin II is secreted in large amounts into the media of Capan-2 cells.

Hajjar's group has made important advances in characterizing annexin II synthesis, export, and membrane binding. Annexin II was shown to be constitutively translocated to the HUVEC extracellular surface within 16 h of biosynthesis (*18*). Furthermore, these authors demonstrated that the HUVEC extracellular surface contained high-affinity and saturable sites for annexin II binding. Last, the possibility that the appearance of AIIt on the outside of cells is due to the release of the protein from dead cells has been ruled out because the extracellular expression of annexins does not correlate the overall cellular expression of various annexins (*19*).

Recently, annexin II was identified as an endothelial cell surface protein that bound t-PA, plasminogen, and plasmin. It was also reported that the ability of annexin II to bind plasminogen and plasmin and also to stimulate plasmin generation required proteolysis of annexin II at Lys-307 (*20*, *21*). Our laboratory reported that AIIt bound t-PA, plasminogen, and plasmin and stimulated t-PA-dependent plasminogen activation about 300-fold (*22*). We also demonstrated that the extracellular p11 subunit was coimmunoprecipitated with extracellular annexin II by an anti-annexin II antibody in HUVEC. Furthermore, immunofluorescence localization experiments established that annexin II and the p11 subunit colocalized on the extracellular surface of HUVEC. These data suggested that most of the annexin II was complexed with the p11 subunit as AIIt, and little, if any, free annexin II was present on the extracellular surface of endothelial cells. If the established role of the p11 as a regulatory subunit and reported stimulation of t-PA-dependent plasminogen activation by annexin II were considered, our results predicted that the p11 subunit might facilitate the p36 subunit-mediated stimulation of t-PA-dependent plasminogen activation.

In this report, we have compared the kinetics of the annexin II- and p11 subunit-mediated stimulation of t-PA-dependent plasminogen activation. Surprisingly, these experiments established that the p11 subunit bound [Glu]-plasminogen and accounted for most of the AIIt-dependent stimulation of t-PA-mediated plasminogen activation.

EXPERIMENTAL PROCEDURES

Materials. [Glu]Plasminogen, [Lys]plasminogen, PAI-1, α_2-antiplasmin, the amidolytic plasmin-specific substrate, Spectrozyme #251 (H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide), and the t-PA-specific amidolytic substrate Spectrozyme # 444 (methyl-D-cyclohexatyrosyl-glycyl-arginine-*p*-nitroaniline acetate) were obtained from American Diagnostica. Human recombinant single-chain t-PA was obtained from Genentech and further purified by chromatography on benzamidine−Sepharose (*23*). Bovine lung AIIt, recombinant AIIt, or recombinant p11 subunit or p36 subunits were prepared as described previously (*22*).

Plasminogen Activation Assay. The kinetics of t-PA-dependent plasminogen activation were determined by measuring the amidolytic activity of the plasmin generated during activation of plasminogen as described previously (*22*). The reaction was performed at 25 °C, in 0.6 mL of a buffer consisting of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$, 1 mM DTT, and 5.6 nM t-PA with the substrate H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide (Spectrozyme #251) at a final concentration of 104 μM. The reaction was initiated by the addition of 0.11 μM [Glu]plasminogen and the reaction progress monitored at 405 nm. In the absence of t-PA, plasmin generation did not occur regardless of the presence or absence of the p36 subunit or AIIt (data not shown).

Amidolytic Activity Assay. Plasmin activity was directly measured with the substrate H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide (Spectrozyme #251) at a concentration of 104 μM, at 25 °C in 0.6 mL of a buffer consisting of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM $CaCl_2$. Typically, the reaction was initiated by addition of plasmin (3.8 nM) and the reaction progress monitored at 405 nm. The t-PA amidolytic activity was measured with 104 μM t-PA-specific amidolytic substrate Spectrozyme #444 under conditions identical to those of the plasmin amidolytic activity assay.

Data Analysis. If a constant rate of plasminogen activation is assumed, the concentration of plasmin increases linearly with time and that of *p*-nitroaniline follows a parabolic curve (*24*, *25*). Initial rates of plasmin generation were calculated using linear regression analysis of plots of A_{405nm} versus time^2 utilizing data points at a low extent of substrate conversion as outlined in ref *22*. Time course data were analyzed according to the equation describing the rate of *p*-nitroanilide (*p*-NA) production from a mixture of t-PA, plasminogen, and H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide, $A_{405nm} = B + Kt^2$, where K is the rate constant for the acceleration of *p*-NA generation and B is the y-intercept. Under our experimental conditions, K was proportional to the initial rate of plasmin formation from plasminogen. Typically, the initial rates of plasmin generation were reported in units (U) of $A_{405nm}/min^2 \times 10^3$. Titration data were analyzed as described previously (*22*) with the four-parameter logistic equation $f = (a - d)/[1 + (x/c)^b] + d$, where a is the asymptotic maximum, b is the slope parameter, c is the value at the inflection point ($A_{0.5}$), and d is the asymptotic minimum.

Production of the p11 Subunit Deletion Mutant. The cDNA of the p11 subunit deletion mutant (lacking the last two lysine residues of the C terminus) was produced by PCR mutagenesis. PCR was performed on the human wild-type p11 cDNA in a Perkin-Elmer System 480 DNA Thermal Cycler using the following primers: 5′-ATGCCATCT-CAAATGGAACACGCCATG-3′ (5′-primer) and 5′-CGG-GATCCCTATCCCTTCTGCTTCATGTG-3′ (3′-primer). The PCR product was digested with *Bam*HI and ligated to *Nde*I-digested, Klenow fragment-filled-in, and *Bam*HI-digested

pAED4.91. The cDNA expression construct, pAED4.91-p11$_{del-KK}$, was confirmed to have Lys-95 and Lys-96 deleted by DNA sequencing and used for transforming BL21(DE3) *Escherichia coli*. The expression of the p11$_{del-KK}$ in *E. coli* was induced by addition of 0.8 mM IPTG in LB media. The p11$_{del-KK}$ mutant was purified as previously described (*13*) with slight modification, in that the fast S column step preceded the fast Q column step and the bound p11$_{del-KK}$ protein was eluted from both columns using a 0 to 2 M NaCl gradient.

Mutant AIIt composed of wild-type p36 and p11$_{del-KK}$ subunits was produced as follows. Both subunits were combined with an equal molar ratio, and the resultant mutant AIIt was isolated by gel permeation chromatography and characterized according to ref *13*. Studies comparing the interaction of the wild-type and mutant AIIt to those of heparin, phospholipid, and F-actin suggested that the structure of the mutant was identical to the wild-type AIIt.

Cell Surface Plasminogen Activation by the p11 Subunit. Renal epithelial 293 cells (purchased from ATCC) were grown in 24-well flasks in M199 media containing 10% heat-inactivated fetal calf serum. The cells were washed twice in PBS containing 1 mM $CaCl_2$ (PBSC) and incubated in PBSC containing 2.0 μM recombinant p11 subunit or 2.0 μM recombinant annexin II for 60 min at 37 °C. The cells were extensively washed with PBSC and incubated at 25 °C in buffer A containing 44 nM [Glu]plasminogen and 104 μM amidolytic substrate. The reaction was initiated by the addition of 8.5 nM t-PA. At timed intervals, aliquots were removed and the reactions quenched with 4 M sodium acetate (pH 3.8), and the absorbance at 405 nm was determined.

Miscellaneous Techniques. The p11 affinity column was constructed by incubation of 10 mg of the p11 subunit with 10 mL of CNBr-activated Sepharose 4B according to the manufacturer's instructions. The AIIt concentration was determined spectrophotometrically using an extinction coefficient A_{280nm} of 0.68 for 1 mg/mL AIIt. The K307T and K328I mutant of the p36 subunit was a generous gift from K. Hajjar (Cornell University Medical College, New York, NY). Recombinant human plasminogen (S741C-fluorescein) was a generous gift from M. E. Nesheim (Departments of Biochemistry and Medicine, Queen's University, Kingston, ON). N-Terminal analysis of plasmin-digested AIIt was performed as follows; 50 nM plasmin and 2 μM AIIt were incubated at 25 °C, in a final volume of 0.6 mL in a buffer consisting of 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $CaCl_2$, and 1 mM DTT. After 2 h, the reaction mixture was subjected to SDS−PAGE followed by transblotting to a PVDF membrane. The appropriate bands were excised and subjected to N-terminal amino acid sequence analysis (*26*). Peptides were synthesized by the University of Calgary Peptide Facility and purified by HPLC.

RESULTS

Stimulation of t-PA-Dependent Plasminogen Activation by AIIt Subunits. To identify the subunit of AIIt involved in stimulation of t-PA-mediated plasminogen activation, we have compared the relative stimulatory activities of the p36 and p11 subunits of AIIt. As shown in Figure 1 and inset A of Figure 1, the basal initial rate of t-PA-dependent activation of [Glu]plasminogen (0.0185 ± 0.0057 U, mean ± SD, $n = 9$) was stimulated about 2-fold to 0.028 ± 0.005 U (mean ± SD, $n = 5$) by annexin II. The p11 subunit stimulated t-PA-dependent activation of [Glu]plasminogen about 45-fold to 0.866 ± 0.20 U (mean ± SD, $n = 9$). By comparison, the native bovine lung AIIt and human recombinant AIIt stimulated the t-PA-dependent activation of [Glu]-plasminogen about 150- and 77-fold, respectively.

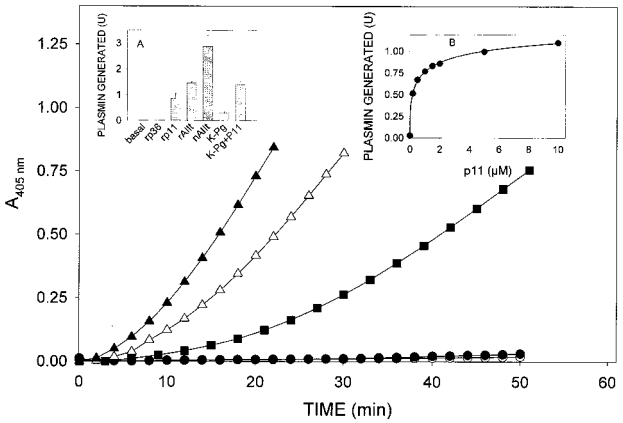

FIGURE 1: Stimulation of t-PA-dependent plasminogen activation by the subunits of the annexin II tetramer. t-PA (5.6 nM) was incubated at 25 °C in buffer A [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM $CaCl_2$] with Spectrozyme #251 substrate (104 μM) in the absence (○, inset A, basal) or presence of purified bovine annexin II (2 μM) (●, inset A, rp36), 2 μM recombinant p11 subunit (■, inset A, rp11), 2 μM recombinant AIIt (△, inset A, rAIIt), or 2 μM bovine lung AIIt (▲, inset A, nAIIt). The reaction was initiated by the addition of 0.11 μM [Glu]plasminogen, and the amidolytic activity of plasmin was monitored at 405 nm as described in Experimental Procedures. (Inset A) Comparison of the rates of plasmin generation calculated from plots of A_{405nm} vs t^2 (mean ± SD): (basal) 0.019 ± 0.006 U ($n = 9$), (rp36) 0.028 ± 0.005 U ($n = 5$), (rp11) 0.866 ± 0.20 U ($n = 9$), (rAIIt) 1.47 ± 0.24 ($n = 4$), and (nAIIt) 2.87 ± 0.43 ($n = 5$). The stimulation of the rates of t-PA dependent [Lys]plasminogen activation in the absence (K-Pg, 0.306 ± 0.05, $n = 3$) and presence of 2 μM p11 subunit (K-Pg+P11, 1.4 ± 0.2, $n = 3$) is also compared. (Inset B) Concentration dependence of the stimulation of t-PA-dependent [Glu]-plasminogen activation by the recombinant p11 subunit. The line through the points is a nonlinear least-squares curve fit of the data points calculated from computer modeling of data to the four-parameter logistic equation (see Experimental Procedures). The line through the points was generated using the following values: $a =$ 1.487 U (asymptotic maximum), $b = 0.425$ (slope parameter), $c =$ 0.95 μM [value at the inflection point ($A_{0.5}$)], $d = 0$ (asymptotic minimum).

Previously, we reported that AIIt stimulated the t-PA-dependent activation of [Lys]plasminogen about 22-fold (*22*). As shown in inset A of Figure 1, the p11 subunit stimulates the t-PA-dependent activation of [Lys]plasminogen about 5-fold. This decreased stimulatory effect of the p11 subunit was partly due to the more rapid rate of t-PA-dependent production of plasmin from [Lys]plasminogen compared to that from [Glu]plasminogen.

The p11 subunit did not affect either the t-PA or the plasmin amidolytic activity (data not shown), therefore, establishing that the p11 subunit directly stimulates the t-PA-dependent activation of [Glu]plasminogen. The stimulation of t-PA-dependent activation of [Glu]plasminogen by the p11 subunit was concentration-dependent, and half-maximal stimulation was observed at about 1 μM p11. At the maximum stimulatory activity, the p11 subunit stimulated the activation of [Glu]plasminogen about 78-fold.

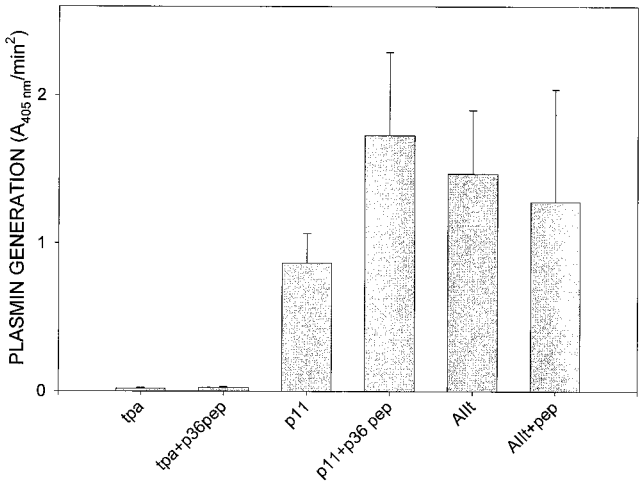

FIGURE 2: Stimulation of p11 activity by a peptide to the p11-binding site of the p36 subunit. t-PA (5.6 nM) was incubated at 25 °C in buffer A with Spectrozyme #251 substrate (104 μM) in the absence (t-PA) or presence (t-PA+p36pep) of a peptide of the p11-binding region of the p36 subunit (Ac-1-S-T-V-H-E-I-L-C-K-L-S-L-E-G-D-15) (100 μM), (p11) 2 μM recombinant p11 subunit, (p11+p36 pep) 2 μM recombinant p11 subunit and 100 μM peptide, (AIIt) 2 μM recombinant AIIt, and (AIIt+pep) 2 μM recombinant AIIt and 100 μM peptide. The reaction was initiated by the addition of 0.11 μM [Glu]plasminogen, and the amidolytic activity of plasmin was monitored at 405 nm as described in Experimental Procedures. The rates of plasmin generation were calculated from plots of A_{405nm} vs t^2 (mean ± SD): (A) 0.019 ± 0.006 U (n = 9), (B) 0.026 ± 0.005 U (n = 3), (C) 0.866 ± 0.20 U (n = 6), (D) 1.73 ± 0.56 U (n = 5), (E) 1.47 ± 0.40 U (n = 5), and (F) 1.28 ± 0.71 U (n = 4).

The first 14 residues of the N terminus of annexin II contain the high-affinity binding site for the p11 protein (reviewed in ref *1*). Interestingly, proteolysis of AIIt by chymotrypsin or during purification of the protein results in the release of the N terminus of the p36 subunit and subsequent release of the p11 subunit (*12*, *27*, *28*). To identify the region(s) of AIIt cleaved by plasmin, AIIt was incubated with plasmin and the proteolyzed protein resolved by SDS−PAGE which demonstrated two p36 subunit bands and a single p11 subunit band. The upper p36 subunit band, consisting of about 80% of the doublet protein, had an M_r identical to that of authentic p36 subunit, while the lower band, consisting of about 20% of the doublet protein, was truncated by about 3 kDa (data not shown). The lower band was subjected to amino acid sequencing which identified the sequence 28-Ala-Tyr-Thr-Gln-Phe-Asp-Glu-Arg-35. This established that plasmin-dependent proteolysis of AIIt resulted in the cleavage of the N terminus of the p36 subunit and the loss of the first 27 residues of the protein. Since the region of AIIt cleaved by plasmin contains the p11-binding site, it is possible that plasmin-dependent proteolysis of AIIt releases the p11 subunit bound to the first 27 amino acids of the N terminus of the p36 subunit.

A peptide of the first 14 amino acid residues of the N-terminal region of the p36 subunit has been shown to bind to the p11 subunit (*29*). We, therefore, investigated the possibility that the binding of the p11 subunit to the p11-binding site of the p36 subunit might affect the activity of the p11 subunit. As shown in Figure 2, the p11-binding peptide of p36 stimulated the t-PA dependent [Glu]plasminogen activation about 2-fold. In contrast, the p11-binding peptide did not stimulate the t-PA-dependent activation of [Glu]plasminogen but did slightly inhibit the AIIt-dependent stimulation of t-PA-dependent activation of [Glu]plasminogen.

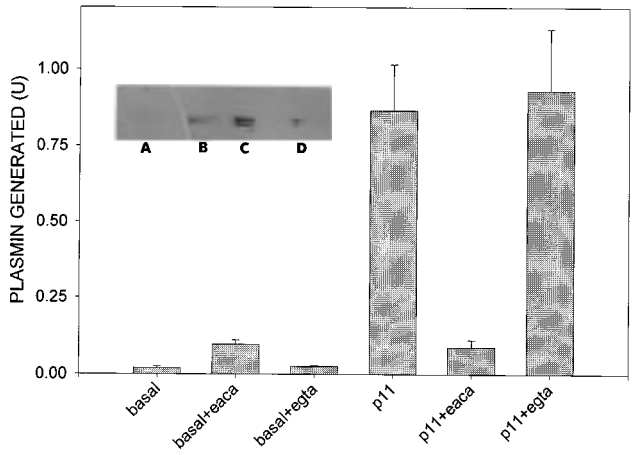

FIGURE 3: Characterization of p11-stimulated t-PA-dependent plasmin generation. Comparison of the rates of plasmin production for reactions conducted as follows: (basal) no addition (0.019 ± 0.006 U, mean ± SD, n = 9), (basal+eaca) 10 mM EACA (0.097 ± 0.014 U, mean ± SD, n = 3), (basal+egta) 5 mM EGTA (0.025 ± 0.003 U, mean ± SD, n = 3), (p11) 2 μM recombinant p11 subunit (0.866 ± 0.15 U, mean ± SD, n = 6), (p11+eaca) 2 μM recombinant p11 subunit and 10 mM EACA (0.088 ± 0.024 U, mean ± SD, n = 3), and (p11+egta) 2 μM recombinant p11 subunit and 5 mM EGTA (0.931 ± 0.20 U, mean ± SD, n = 3). (Inset) A p11 affinity column was equilibrated with 10 mM phosphate (pH 7.3) and 100 mM NaCl. After application of [Glu]plasminogen, the column was extensively washed and eluted with 10 mM EACA (A), 20 mM EACA (B), 50 mM EACA (C), and 100 mM EACA (D). Fractions were analyzed by SDS−PAGE.

Characterization of the Activation of [Glu]Plasminogen by the p11 Subunit. The stimulation of the t-PA-dependent activation of [Glu]plasminogen by the p11 subunit suggested that p11 and [Glu]plasminogen formed a complex. This was directly tested by application of [Glu]plasminogen to a p11 affinity column. As shown in the inset of Figure 3, [Glu]-plasminogen bound to the p11 affinity column and was eluted with the lysine analogue ϵ-amino-*n*-caproic acid (EACA). These data established that the p11 subunit binds [Glu]-plasminogen and suggested that the lysine-binding kringle domains of [Glu]plasminogen were involved in the binding of the p11 subunit.

We further investigated the possibility that the p11-dependent stimulation of t-PA activity might be due to the interaction of the lysine binding sites of t-PA or [Glu]-plasminogen with lysine residues of p11. As shown in Figure 3, 10 mM EACA stimulated the rate of t-PA-dependent activation of [Glu]plasminogen about 5-fold. In contrast, EACA dramatically inhibited the p11-dependent stimulation of t-PA-dependent [Glu]plasminogen activation. These data further imply that the stimulation of the rate of activation of [Glu]plasminogen by the p11 subunit occurs via lysine binding site-dependent interactions.

We have established that the AIIt-mediated stimulation of t-PA-dependent activation of [Glu]plasminogen is Ca^{2+}-independent (*22*). Similarly, we have found that the rates of the p11-mediated stimulation of t-PA-dependent activation of [Glu]plasminogen in a Ca^{2+}-containing buffer or Ca^{2+}-free buffer with EGTA are similar (Figure 3).

The p11 Subunit Protects Plasmin from Inactivation by α_2*-Antiplasmin.* The primary physiological inhibitor of

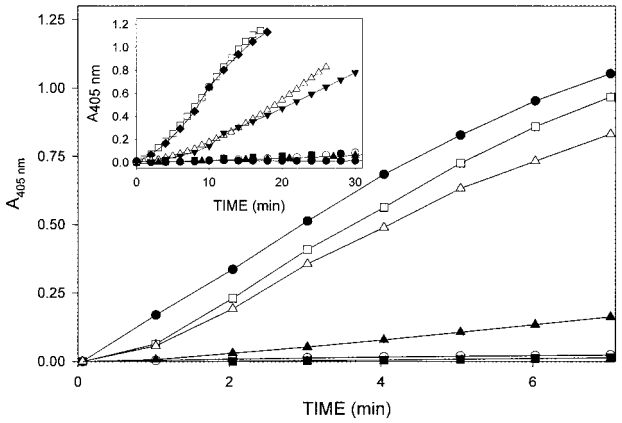

FIGURE 4: Effects of PAI-1 and α_2-antiplasmin on AIIt and p11 subunit activity. Plasmin (105 nM) was incubated at 25 °C in buffer A and in the absence (●) or presence (○) of 210 nM α_2-antiplasmin. In other experiments, plasmin was preincubated in the presence of recombinant p11 subunit (□) or bovine lung AIIt (△). After 5 min, the mixture was adjusted to 210 nM α_2-antiplasmin and the reaction was initiated by the addition of 104 μM Spectrozyme #251 substrate. In other reactions, the recombinant p11 subunit or bovine lung AIIt was preincubated with α_2-antiplasmin for 5 min followed by addition of plasmin (■ and ▲, respectively). The amidolytic activity of plasmin was monitored at 405 nm as described in Experimental Procedures. (Inset) Comparison of the rates of t-PA-dependent plasmin production. T-PA-dependent plasminogen activation was measured (28 nM t-PA) in the absence (○) (0.019 ± 0.006 U, mean ± SD, $n = 9$) or presence of 58 nM PAI-1 (●) (0.097 ± 0.014 U, mean ± SD, $n = 3$). Other reaction mixtures contained t-PA and 2 μM recombinant p11 subunit (△) (0.025 ± 0.003 U, mean ± SD, $n = 3$) or t-PA, 2 μM recombinant p11 subunit, and 58 nM PAI-1 (▲) (0.866 ± 0.15 U, mean ± SD, $n = 6$). The t-PA-dependent activation of plasmin production was also examined in the presence of 2 μM bovine lung AIIt (□) (0.088 ± 0.024 U, mean ± SD, $n = 3$) or 2 μM bovine lung AIIt and 58 nM PAI-1 (■) (0.931 ± 0.20 U, mean ± SD, $n = 3$). In other experiments, AIIt (◆) or p11 (▼) was preincubated with t-PA for 5 min before addition of PAI-1 and the rate of t-PA-dependent plasmin production was determined.

plasmin is α_2-antiplasmin (*30*). Although our data clearly established that AIIt and its p11 subunit could stimulate the t-PA-dependent conversion of plasminogen to plasmin (Figure 1 and ref *22*), it is unclear if the plasmin generated by this mechanism remains bound to these proteins. As shown in Figure 4, plasmin activity was dramatically inhibited by α_2-antiplasmin. In contrast, in the presence of either AIIt or the p11 subunit, plasmin activity was unaffected by α_2-antiplasmin. However, the protection of plasmin from inactivation by α_2-antiplasmin required preincubation of plasmin with AIIt or the p11 subunit. Preincubation of α_2-antiplasmin with AIIt or the p11 subunit prior to addition of plasmin did not protect plasmin from inactivation.

PAI-1 is an important inhibitor of the t-PA-dependent activation of plasminogen. We, therefore, examined the effect of PAI-1 on the AIIt- and p11-dependent stimulation of t-PA-dependent activation of [Glu]plasminogen. As shown in the inset of Figure 4, AIIt and the p11 subunit block the PAI-1-dependent inhibition of [Glu]plasminogen activation by t-PA when t-PA was preincubated with AIIt or the p11 subunit. In contrast, preincubation of PAI-1 with AIIt or the p11 subunit prior to addition of t-PA did not protect t-PA from inactivation by PAI-1. Therefore, our results imply that t-PA binds to the p11 subunit of AIIt and is then protected from inactivation by PAI-1.

Table 1: Modulation of p11- and AIIt-Stimulated t-PA-Dependent Plasminogen Activation by the p11 C-Terminal Peptide[a]

addition	p11 subunit residual activity (%)	AIIt residual activity (%)
C-terminal peptide	56 ± 7	37 ± 3
HBS peptide	107 ± 27	ND
p11 peptide	96 ± 3	ND

[a] t-PA (5.6 nM) was incubated at 25 °C in buffer A [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM $CaCl_2$] with Spectrozyme #251 substrate (104 μM) in the presence or absence of 100 μM peptide and in the absence or presence of 2 μM recombinant p11 subunit or 2 μM bovine lung AIIt. The reaction was initiated by the addition of 0.11 μM [Glu]plasminogen, and the amidolytic activity of plasmin was monitored at 405 nm as described in Experimental Procedures. Residual activity refers to the percentage of the rate of p11- or AIIt-stimulated t-PA-dependent plasminogen activation remaining after addition of peptide. The peptides used in this study were the p11 C-terminal peptide (85-Y-F-V-V-H-M-K-Q-K-G-K-K-96), the peptide of the heparin binding site of the p36 subunit (HBS peptide, 300-L-K-I-R-S-E-F-K-K-K-Y-G-K-S-L-Y-Y-316), and the peptide of a region of p11 (21-D-K-G-Y-L-T-K-E-D-L-R-V-L-M-E-K-E-K-38). Data are presented as the mean ± SD ($n = 3$).

Role of the C-Terminal Lysines of the p11 Subunit. Plasminogen is known to bind to its extracellular binding sites by a mechanism involving interaction of the C-terminal lysine residues of the binding sites with the kringle domains of plasminogen (*31*). We initially observed that treatment of AIIt with carboxypeptidase B, an enzyme that selectively removes C-terminal lysine residues, resulted in the loss of about 93% of the ability of AIIt to stimulate t-PA-dependent plasminogen activation (data not shown). A comparison of the last 10 amino acid residues of the C terminus of the p36 subunit (329-A-L-L-Y-L-C-G-G-D-D-338) with that of the p11 subunit C terminus (87-V-V-H-M-**K**-Q-**K**-G-**K**-**K**-96) indicated that only the p11 subunit has the prerequisite C-terminal lysine residues. The presence of lysine residues at the last two positions of the C terminus of the p11 subunit suggested the possibility that these C-terminal lysine residues might participate in t-PA-dependent plasminogen activation. We initially tested the possibility that the C-terminal domain of the p11 subunit of AIIt participated in the stimulation of t-PA-dependent plasminogen activation. A peptide of the C-terminal domain of the p11 subunit was prepared and tested as a potential inhibitor of p11-stimulated, t-PA-dependent plasminogen activation. As shown in Table 1, the p11 C-terminal peptide (85-Y-F-V-V-H-M-K-Q-K-G-K-K-96) inhibited both p11- and AIIt-stimulated, t-PA-dependent [Glu]plasminogen activation.

We also directly examined the function of the C-terminal residues of the p11 subunit by producing a p11 deletion mutant that lacked the last two C-terminal lysine residues. As shown in Figure 5, the mutant $p11_{del-KK}$ subunit stimulated t-PA-dependent plasminogen activation only 7-fold compared to a 45-fold stimulation by the wild-type protein.

We have also directly evaluated the contribution of the C-terminal lysines of the p11 subunit of AIIt to the ability of AIIt to stimulate t-PA-dependent plasminogen activation. We prepared a mutant recombinant AIIt that consisted of wild-type p36 subunits and the mutant $p11_{del-KK}$ subunit and examined the ability of this mutant AIIt to stimulate t-PA-dependent [Glu]plasminogen activation. As shown in Table

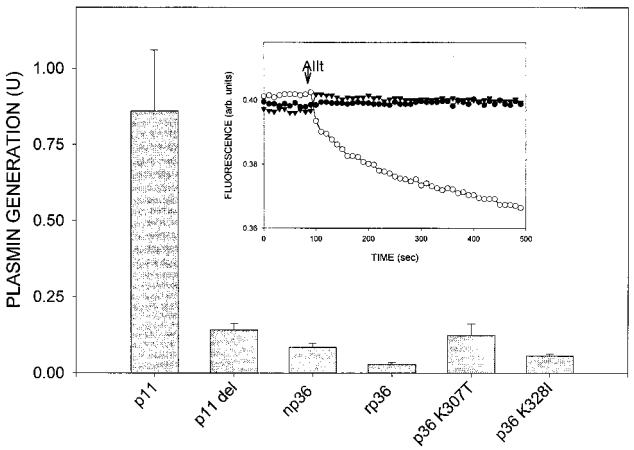

FIGURE 5: Role of p36 and p11 subunit lysines in t-PA-dependent plasmin generation. The rates of t-PA-dependent plasmin generation were determined as described in Figure 1. Incubations were performed in the presence of 2 μM recombinant p11 subunit (p11), 2 μM recombinant $p11_{del-KK}$ subunit mutant (p11 del), 5 μM bovine lung p36 subunit (np36), 5 μM wild-type recombinant p36 subunit (rp36), 5 μM recombinant p36 subunit (K307T) mutant (p36 K307T), and recombinant p36 subunit (K328I) mutant (p36 K328I). (Inset) [Glu]Plasminogen(S741C-fluorescein) (0.11 μM) was incubated at 25 °C in buffer A, and the fluorescence intensity was measured at excitation and emission wavelengths of 495 and 535 nm, respectively. After about 100 min, 2 μM wild-type recombinant AIIt (○), mutant recombinant AIIt consisting of wild-type recombinant p36 subunit and the recombinant $p11_{del-KK}$ subunit (▼), or a dilution control (●) was added to the reaction mixture.

Table 2: Effect of an AIIt Deletion Mutant (last two C-terminal lysine residues deleted from the p11 subunit) on the Stimulation of t-PA-Dependent Activation of [Glu]Plasminogen[a]

[AIIt deletion mutant] (μM)	residual activity (%)
0.5	10.4 ± 2.2
1.0	12.7 ± 5.8
2.0	12.2 ± 3.2

[a] t-PA-dependent plasminogen activation was measured as described in Figure 1. Assays were conducted in the presence of wild-type or mutant recombinant AIIt (consisting of the wild-type recombinant p36 subunit and the mutant recombinant $p11_{del-KK}$ subunit). Results are expressed as the mean ± SD for at least three experiments. The residual activity is defined as the percentage of the mutant AIIt activity compared to the wild-type recombinant AIIt activity.

2, the mutant AIIt possessed only about 12% of the wild-type recombinant AIIt activity.

We recently showed that direct addition of AIIt to [Glu]-plasminogen(S741C-fluorescein) resulted in a rapid decrease in [Glu]plasminogen(S741C-fluorescein) fluorescence (*22*). This suggested that the binding of AIIt to plasminogen induced a conformational change in the microenvironment in the vicinity of the active site of plasminogen. As shown in the inset of Figure 5, in contrast to wild-type AIIt, the mutant AIIt failed to induce a conformational change in [Glu]-plasminogen(S741C-fluorescein). This establishes the importance of the C-terminal lysines of the p11 subunit in both the binding to plasminogen and stimulation of t-PA-dependent plasminogen activation.

Although annexin II is a weak stimulator of t-PA-dependent plasminogen activation (Figure 1), it is possible that the binding of annexin II to p11 could enhance the activity of the annexin II. In other words, the p36 subunit of AIIt could have an enhanced activity compared to annexin

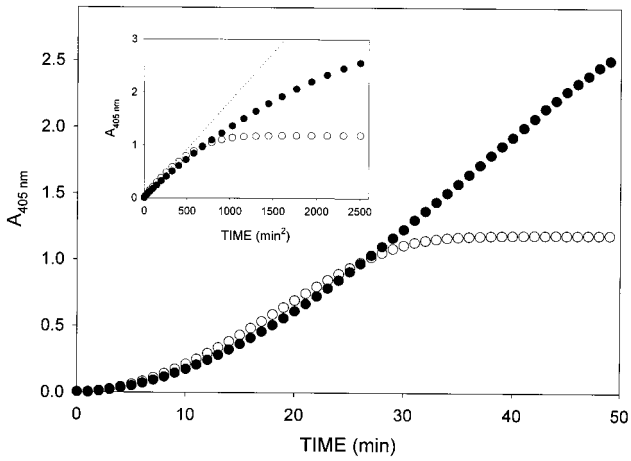

FIGURE 6: Effects of substrate concentration on the AIIt-mediated stimulation of t-PA-dependent plasminogen activation. The rates of t-PA-dependent plasmin production were compared in a reaction mixture containing buffer A [50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM $CaCl_2$], t-PA (5.6 nM), native bovine lung AIIt (2 μM), and either 104 (○) or 520 μM (●) Spectrozyme #251 substrate. The reaction was allowed to proceed until substrate depletion caused the reaction rate to decrease. The inset shows the linear regression analysis of plots of A_{405nm} vs t^2.

II. Therefore, we sought to identify the amino acid residues in annexin II that were critical for stimulation of t-PA-dependent plasminogen activation. Mutation of these residues and inactivation of annexin II activity should allow production of a mutant AIIt comprised of the mutant p36 subunit and the wild-type p11 subunit. This mutant would allow direct analysis of the contribution of the p36 subunit to the stimulation of t-PA-dependent plasminogen activation by AIIt. It has been suggested that K307 of annexin II is an essential residue for the binding of plasminogen to annexin II (*32*). We, therefore, compared the stimulatory activity of wild-type recombinant annexin II with that of K307T and K328I recombinant annexin II mutants. As shown in Figure 5, these amino acid substitutions did not dramatically affect the stimulatory activity of annexin II.

Virtually all the interactions of t-PA, plasminogen, and effectors involve the lysine-binding sites on both proteins. Our data established the importance of the C-terminal lysines of the p11 subunit of AIIt in the stimulation of t-PA-dependent plasminogen activation. However, the substrate, H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide (Spectrozyme #251), used in these experiments to measure plasmin activity, also contains a C-terminal lysine. We, therefore, investigated the possibility that AIIt might compete for the substrate-binding site of plasmin. As shown in Figure 6, stimulation of the plasminogen activation rate by AIIt was not influenced by a 5-fold range of substrate concentrations. It was also interesting to note that the initial rates of the reaction were linear over a wide range of substrate (inset of Figure 6). The linearity of the initial rates of acceleration of plasmin production proves that AIIt does not compete with the amidolytic substrate for the substrate-binding site of plasmin.

Cell-Associated p11 Subunit Activates [Glu]Plasminogen. We have established that the p36 and p11 subunits of AIIt colocalize on the extracellular surface of HUVEC and that AIIt bound to the extracellular surface stimulates t-PA-dependent plasminogen activation (*22*). Although the major-

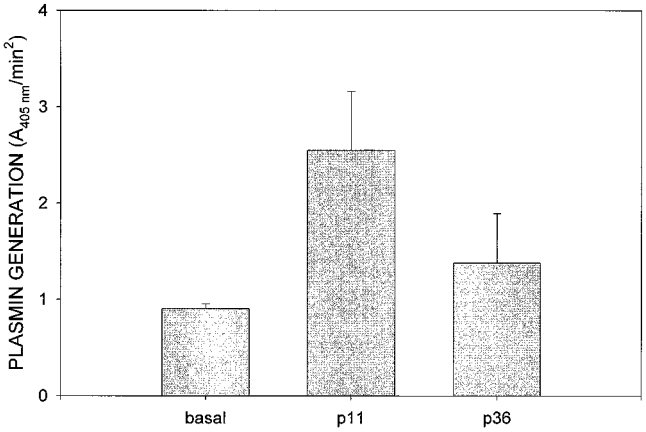

FIGURE 7: Stimulation of plasmin generation by the cell-associated p11 subunit. Human 293 cells were incubated with PBS containing 1 mM $CaCl_2$ in the absence (basal) or presence of 2 μM p11 subunit (p11) or 2 μM p36 subunit (p36). The cells were washed with PBS containing 1 mM $CaCl_2$, and the rates of plasmin generation were determined as described in Figure 1.

ity of the p11 subunit is colocalized with annexin II, a small amount of free p11 subunit appeared to be associated with the extracellular surface of HUVEC. We, therefore, examined the possibility that the free p11 subunit, bound to the extracellular surface of the plasma membrane, might also stimulate t-PA-dependent plasminogen activation. Human 293 epithelial cells were used in these experiments because these cells do not have appreciable amounts of annexin II on their extracellular surface (*8*). As shown in Figure 7, the p11 subunit bound to 293 epithelial cells was capable of stimulating t-PA-dependent plasminogen activation about 3-fold. Since the p11 subunit, unlike annexin II, cannot bind phospholipid, these results also suggest that the p11 subunit may bind to an extracellular membrane protein.

DISCUSSION

Plasminogen-binding sites on cells are heterogeneous in nature. Proteins, nonproteins, and glycosaminoglycans can function as plasminogen-binding sites (*33*−*39*). The fundamental characteristics of cellular receptors for plasminogen are their low affinity (0.1−2.0 μM), high density (10^6−10^7 sites per cell), and ubiquitous distribution (*31*, *40*, *41*). Analysis of the binding isotherms from a variety of different cells has suggested the existence of a single class of low-affinity plasminogen binding sites on all cells examined. Interestingly, the interaction of plasminogen with its cell surface receptor(s) is inhibited by lysine analogues or by pretreatment of the cells with carboxypeptidase B, an enzyme that removes C-terminal amino acid residues. Of the total plasminogen-binding sites on many cells, about one-half are blocked by treatment of cells with carboxypeptidase B (*31*). This implies that many of the cellular plasminogen-binding sites are proteins with C-terminal lysine residues that bind to the kringle domains of plasminogen. It has also been demonstrated that the specific subset of plasminogen binding proteins with exposed C-terminal lysine residues on the cell surface are the sites of cellular plasminogen activation. This has been interpreted to imply that the activation of plasminogen by its physiological receptors involves the interaction of the lysine-binding sites of plasminogen with the C-terminal lysine residues of plasminogen receptors (*31*).

We observed that the isolated p11 subunit of AIIt directly stimulated t-PA-dependent plasminogen activation. The presence of two lysine residues at the C terminus of the p11 subunit suggested the possibility that this region of the p11 subunit might participate in stimulation of t-PA-dependent plasminogen activation. Consistent with this possibility, a peptide to the C-terminal domain of the p11 subunit inhibited the stimulation of t-PA-dependent plasminogen activation by both AIIt and the p11 subunit. To directly test the role of the p11 subunit C-terminal lysines in plasminogen regulation, we produced a mutant AIIt consisting of the recombinant wild-type p36 subunit and the $p11_{del-KK}$ subunit. The AIIt mutant retained only about 12% of the activity of the wild-type recombinant AIIt. Therefore, these data establish the critical role that the C-terminal lysines of the p11 subunit play in the stimulation of t-PA-dependent plasminogen activation by AIIt. Moreover, these data also establish that the p36 subunit of AIIt plays only a minor role in the stimulation of t-PA-dependent plasminogen activation by AIIt.

We also showed that in contrast to wild-type recombinant AIIt, the recombinant AIIt deletion mutant failed to induce a conformational change in [Glu]plasminogen(S741C-fluorescein). These observations are consistent with the p11 subunit of AIIt playing an essential role in the binding of AIIt to plasminogen. We have also observed that plasminogen binds to the isolated recombinant p11 subunit and that the binding is reversed by EACA. Moreover, the stimulation of t-PA-dependent plasminogen activation by AIIt and by the isolated recombinant p11 subunit is blocked by EACA (*22*). Collectively, these results indicate that the interaction between plasminogen and AIIt is due to the interaction of plasminogen kringle domains with lysine residues of AIIt. The lysine residues involved in the interaction between AIIt and plasminogen are contributed by the p11 subunit C terminus. Furthermore, since the binding of the p11-binding site peptide of the p36 subunit (the N-terminal domain of the p36 subunit) to the p11 subunit further enhanced the stimulatory activity of the p11 subunit, the p36 subunit functions to stimulate the activity of the p11 subunit.

The enzymatic activity of free plasmin in the plasma has an extremely short half-life, due to its rapid inactivation by α_2-antiplasmin. In contrast, cell-associated plasmin is partially protected from inactivation (*30*, *42*). Our observation that the p11 subunit plays an essential role in the protection of plasmin and t-PA from inactivation by α_2-antiplasmin and PAI-1, respectively, is consistent with our proposed role for AIIt and not annexin II as a physiological receptor for plasminogen.

The role of annexin II in the stimulation of t-PA-dependent plasminogen activation is at present unclear. For example, it is uncertain whether sufficient free annexin II exists on the extracellular surface of the endothelial cell to account for a significant amount of plasminogen activation. It is also unclear if annexin II (or the p36 subunit of AIIt) requires processing before it can participate in plasminogen activation. It was originally suggested that annexin II but not the p11 subunit was present on the surface of endothelial cells (*18*). However, we demonstrated by immunofluorescence colocalization and immunoprecipitation studies that the majority of annexin II was present as the p36 subunit of AIIt on the surface of HUVEC and, therefore, AIIt was the predominant form of the protein (*22*). If AIIt is considered a much more potent stimulator of t-PA-dependent plasminogen activation,

it is likely that free annexin II makes only a minor contribution to t-PA-dependent plasminogen activation on endothelial cells.

The observation that the annexin II-mediated stimulation of t-PA-dependent plasminogen activation in vitro is inhibited by pretreatment of annexin II with carboxypeptidase B (*20*) is difficult to reconcile with the fact that annexin II does not possess a C-terminal lysine residue. To explain these results, it has been suggested that the binding of plasminogen by annexin II requires proteolysis of annexin II by a plasmin-like serine protease (*20*). It was also reported that a K307T annexin II mutant, expressed in a eukaryotic expression system, was inactive compared to a K328I annexin II mutant. Therefore, it was proposed that the putative serine protease hydrolyzed annexin II between Lys-307 and Arg-308 and the removal of the new C-terminal Lys-307 by carboxypeptidase B resulted in the inactivation of the annexin II. This proposal is relevant to our analysis since it suggests the possibility that proteolysis of the p36 subunit of AIIt might activate the subunit. Under these conditions, the p36 subunit might assume a more prominent role in the stimulation of t-PA-dependent plasminogen activation by AIIt.

We have directly tested the possibility that the p36 subunit is activated by proteolysis. First, although extensive incubation of AIIt or annexin II with plasmin or other proteases such as trypsin or chymotrypsin does result in proteolysis, the site of cleavage of annexin II by these proteases is the N terminus. For example, the incubation of annexin II with high concentrations of plasmin results in the cleavage of the protein and the loss in M_r of about 3000 Da. N-Terminal analysis indicated that plasmin cleaved the peptide bond between Lys-27 and Ala-28. Since the loss of the amino acids by N-terminal cleavage exactly accounts for the loss in molecular mass of the protein observed by SDS−PAGE analysis of the proteolyzed protein, it is unlikely that plasmin-dependent cleavage of the C terminus occurred. We cannot, however, rule out the possibility that a protease exists that cleaves annexin II at the C terminus. However, other laboratories have reported the proteolysis of annexin II at the N terminus (*12*, *27*, *28*). Second, the processing of annexin II at the C terminus and exposure of a new C-terminal Lys-307 would result in the loss of 30 amino acids and a loss in molecular mass of about 3700 Da. However, SDS−PAGE analysis of the annexin II immunoprecipitated from surface-biotinylated HUVEC demonstrated that the extracellular (biotinylated) annexin II had an M_r identical to that of intact annexin II (*22*), indicating that in vivo the majority of annexin II was not proteolyzed.

Plasminogen binding to cell surfaces results in enhanced plasminogen activation, localization of the proteolytic activity of plasmin on cell surfaces, and protection of plasmin from α_2-antiplasmin. Our results suggest that AIIt may be an important regulator of plasminogen activation and the p11 subunit provides the prerequisite C-terminal lysines for plasminogen binding and activation.

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